REMARKS

Favorable reconsideration is respectfully requested in view of the foregoing amendments and the following remarks.

I. CLAIM STATUS AND AMENDMENTS

Claims 15-34 are pending in this application.

Claims 15-19, 23-25 and 29-32 were examined on the merits and stand rejected.

Claims 20-22, 26-28, 33, and 34 were withdrawn as non-elected subject matter.

Claim 15 has been amended in a non-narrowing manner to better conform to US practice and form for method claims. Further support can be found in the disclosure, for example, at page 6, lines 15-19, all of page 7, and original claim 1.

Claims 16-19, 24, and 29-32 have been amended to make minor editorial revisions to better conform to US claim form. Such revisions are non-substantive and not intended to narrow the scope of protection. Such revisions include: revising the beginning of the claims to recite "A" or "The" and revising the claim language to provide proper antecedent basis throughout the claims.

Applicant notes that the above claim amendments are intended to address matters of form only as they are not intended to affect the scope of the claims. Accordingly, if the next

Office Action on the merits includes a new rejection of one or more claims, the Action must be non-final.

Applicant is submitting the present Amendment without prejudice to the subsequent prosecution of claims to some or all of the subject matter which might be disclaimed by virtue of this response (although none is believed to be), and explicitly reserve the right to pursue some or all of such subject matter, in Divisional or Continuation Applications.

Applicant thanks the Examiner for the careful examination of this case and respectfully request reexamination and reconsideration of the case, as amended. Below Applicant addresses the rejections in the Office Action and explains why the rejections are not applicable to the pending claims as amended.

II. INFORMATION DISCLOSURE STATEMENT

In item 2 on page 2 of the Office Action, the Office did not consider JP 2000-83656 and JP 2002-537849 in the IDS of December 13, 2005, on the basis that Applicant did not provide the documents to the Office.

Applicant respectfully disagrees. Copies of the references should have been forwarded to the USPTO by the International Search Authority pursuant to the trilateral agreement between the USPTO, EPO and JPO. Applicant also notes that an English language version of the International Search

Report (citing these references) was submitted with the December 13, 2005 IDS. Accordingly, the references should be of record at the USPTO, and thus they should be officially considered.

Nonetheless, Applicant is submitting an English translation of the description, claims and Abstract of JP 2000-83656 for official consideration. Also, please note that JP 2002-537849 has a US counterpart that is US2003/0082152A1 as noted on the face of JP 2002-537849. Pursuant to US practice, the US counterpart should be sufficient in order to receive official consideration of JP 2002-537849. Thus, kindly consider JP 2000-83656 and JP 2002-537849 in the IDS of December 13, 2005, and return an Examiner-initialed copy of PTO-1449 form indicating such.

III. FOREIGN PRIORITY

In item 3 on page 8 of the Office Action, the Examiner acknowledged receipt of the certified foreign priority document but not receipt of an English translation thereof. The Examiner contends that Applicant should submit a verified English translation of the certified priority documents to perfect the foreign priority claim. However, pursuant to US practice, the verified English translation is only necessary to overcome intervening prior art, which does not appear to be the case here.

Thus, it is believed that the requirements to establish foreign priority have been met, and as such, Applicant is

entitled to his foreign priority claim to Japanese application no. 2003/170011, filed June 13, 2003. Kindly acknowledge such in the next Office Action.

IV. ANTICIPATION REJECTION

Claims 15, 18, 23, and 24 were rejected under 35 U.S.C. § 102(b) as anticipated by PARK (Bone, 1999, 24: 549-554) as evidenced by LECOEUR (Biomaterials, 1997, 18: 989-993) for the reasons in item 5 on page 9 of the Office Action.

This rejection is respectfully traversed.

It is well established that to anticipate a claim, a cited prior art reference must disclose or suggest each and every element of the claimed invention. *See*, M.P.E.P., Eighth Ed., Rev. 6 (September 2007) at § 2131.

Amended independent claim 15 recites:

> A method of acquiring a cell having other functions, said method comprising inducing transdifferentiation of a preadipocyte cell line, wherein said preadipocyte cell line is obtained by dedifferentiating a mature adipocyte derived from a fat tissue and expresses early markers of osteogenesis, myogenesis or adipognesis.

It is respectfully submitted that the rejection fails, because PARK does not disclose or suggest each and every element of claim 15, namely, inducing transdifferentiation of a preadipocyte cell line obtained by dedifferentiating a mature adipocyte.

The Office relies on PARK as allegedly teaching a method of "isolating, cultivating, and cloning mature adipocytes from human bone marrow; and the cloned mature adipocytes are further dedifferentiated to fibroblast-like fat cells (i.e., pre-adiopocytes."

However, PARK fails to teach that for which it is offered.

Applicant respectfully submits that the cell used for transformation in PARK is a fibroblast derived from brown adipocyte, which was differentiated from a bone marrow stromal cell. As such, the fibroblast cell in PARK is different from the preadipocyte in the method of independent claim 15.

The cell obtained as an adipocyte by PARK was included in a suspended fraction obtained after centrifugation treatment of bone marrow samples. As such, it would have been necessary to first utilize collagenase processing and filtration by a mesh having pore size of approximately 250 μm in order to isolate an adipocyte from the suspended fraction. However, PARK does not carry out such steps. Consequently, it is believed that the cell used by PARK is not an isolated adipocyte, but a mixture of adipocyte and bone marrow stromal cell.

PARK appreciated that the cells present in the floating low-density layer of human bone marrow are mainly adipocytes and preadipocytes. Single adipocytes are present together with adipocytes associated in conglomerates with fibroblastic cells.

See, for instance, the discussion of the PARK experiments on page 553. The various stromal cells present in bone marrow tissue form clusters with adipocytes, and these clusters are likely to exist in the "cells contained in a suspended fraction after centrifugation of the bone marrow samples", which is the "adipocyte" of PARK.

However, the prior art as evidenced by JP 2000-83656 in the IDS filed December 13, 2005, shows that in order to obtain a unilocular adipocyte, collagenase processing is needed, which includes multiple rounds of centrifugation and a filtration mesh of about 250μm.

However, as stated in the traversal to the Restriction Requirement in the response filed February 29, 2008, PARK fails to disclose or suggest any collagenase treatment and filtration. If such were used, the methods would have been described (or references thereto would have been given) so that others could follow the methods and reproduce the results. Again, such procedures were not disclosed in PARK. The skilled artisan, upon reading PARK, would understand that no such treatment took place in the process of PARK. Further, it is apparent that PARK does not disclose checking the suspended fraction obtained to see if it is composed solely of adipocytes.

As such, the skilled artisan, upon reading PARK, would understand that the cell in PARK is not a preadipocyte as required in claim 15.

Next, Applicant will explain why collagenase treatment and filtration are necessary, because of the tissue structure of bone marrow to obtain a single fraction of adipocyte. Indeed, collagenase treatment and filtration are essential for the reasons discussed below:

The structure of the tissue of bone marrow includes reticular tissue that forms stromata and the hematopoietic cells fill the mesh of it. Reticular tissue consists of reticular cells and reticular fibers, thereby forming a microenvironment for induction of differentiation and modulation of hematopoietic cells. Reticular cells include fibroblast, preadipocyte, cells surrounding blood vessels, smooth muscle cells, bone marrow stem cells, and so on, that produce reticular fibers (collagens). It is known that some of the reticular cells, preadipocytes, differentiate into adipocytes and fill the gap within the pith cavity when hematopoiesis is inactive. Thus, there must be stem cells which can differentiate into preadipocytes and adipocytes. These cells within the reticular tissue cannot be distinguished from one another because they all have similar shapes. Consequently, in order to isolate the adipocyte in the reticular tissue of bone marrow, it is necessary to fractionate the cells by digesting the reticular fibers with collagenase.

Collagenase is the enzyme used to digest the extracellular matrix which connects the various types of cells within adipose tissue. All tissue consists of a variety of

different cell types but the digestive enzyme is required to digest the extracellular matrix to enable isolation of the cells from the tissue varies depending on the tissue. The isolation of the cells requires the follow up filtration step to remove undigested tissue. Centrifugation is used to force the adipocytes more to the upper layer fraction because lipid droplets abundant in the cytoplasm cause buoyancy while the other cells precipitate. Thus, the pure adipocyte can be isolated. Ceiling culture, as seen in the present application, enables floating adipocytes exclusively to be cultured. One skilled in the art at the time the invention was made was well aware that, if the enzyme digestion and filtration process is not followed, the adhesive cells recovered will contain various types of cells from the bone marrow tissue, and not the preadipocyte line as claimed.

The obtaining of fibroblast-like adipocytes from the cell suspension containing only mature adipocytes is allegedly carried in PARK in sidewell plates. One skilled in the art would be aware that it is impossible for the suspended cells to attach to the bottom of the wells because they remain suspended within the upper portion of the culture and medium due to the large amount of lipid droplets in cytoplasm.

By contrast, in the present application, the Applicant performed ceiling culture to obtain the adipocytes. The

adipocytes attach themselves to the surface of the dish allowing them to display proliferative capacity.

PARK, however, does not take this measure. Accordingly, the cell cluster containing adipocytes and bone marrow stromal cells, when cultured in six well culture dishes, is going to lead to fibroblasts falling off and there is doubt, therefore, as to the conclusions of PARK. These fibroblasts from bone marrow stromal cells could be ones which are subjected to later processing by PARK.

In order to obtain the fibroblast-like cells, PARK used a culture medium in which the fraction was induced to differentiate into an adipocyte. However, a culture medium for inducing differentiation usually has the potential of maintaining the function of the adipocyte and suppressing dedifferentiation. There is a contradiction here in using a culture medium to induce differentiation in order to dedifferentiate the adipocyte. Yet it makes sense if the starting point is pluripotent stem cells, which will differentiate into adipocytes.

Based on such, one skilled in the art would clearly doubt the disclosure of PARK as confirmation from bone marrow stromal cells.

Furthermore, contrary to the Office's position, there is no disclosure that the cell line used in PARK expresses an early marker of osteogenesis, myogenesis or adipogenesis. One skilled in the art has read reports in a number of publications

that, when using conventional preadipocyte strains, the early differentiation marker genes for adipogenesis are expressed only after induction of differentiation, but not before induction. One skilled in the art at the time of filing the application was of the opinion that the adipocyte maintained a function by expressing adipocyte specific genes. It is respectfully submitted that the absence of any mention of the early marker genes in PARK confirms that the identity of their cells remains unknown.

It is quite likely that the cells originated from the bone marrow stromal cells. If that is indeed the case, it is not at all surprising that their adipocyte derived cells were induced to differentiate into adipocyte by the culture medium for induction of differentiation into adipocyte. Bone marrow stromal cell populations contain pluripotent stem cells.

Applicant again respectfully submits that this lack of clarity and unpredictability as to the cells actually used by PARK is such that the reference fails to teach the dedifferentiation of mature adipocytes derived from bone marrow followed by the transdifferentiation as required in claim 15. Indeed, at page 10, lines 5-6 of the Office Action, the Office acknowledges such and states "Park et al. do not teach deriving their pre-adipocytes from the dedifferentiation of mature adipocytes isolated from subcutaneous fat tissue."

Applicant further notes that the authors of the PARK paper tried to prove that the cells obtained were derived from adipocytes by using anti-AP2 antibody and Oil red O staining. In fact, differentiation to adipocyte can be confirmed by assessing the expression of *ap2* gene that is known to be a late marker gene of the differentiation to adipocyte or by performing Oil red O staining of lipid adipocytes.

The cells obtained by PARK expressed those markers because they had been cultured in a culture medium which induces the differentiation to adipocyte. That is, these cells were the cells which had already been differentiated into adipocyte and possessed the function of adipocytes. In these differentiated cells, the expression of genes and proteins which are specifically expressed in other types of cells (bone, muscle and chondrocyte, for example) is suppressed. In order to differentiate into another cell type, the function of the adipocyte must be turned off by dedifferentiation.

However, there appears to be no such measure taken for the cells obtained by PARK. Thus, Applicant again respectfully submits that the cells obtained in PARK must be <u>different</u> from the preadipocyte strain of the method of claim 15. Indeed, the cells are most likely the cells derived from multi-locular adipocytes that have been induced by differentiation of bone marrow stromal cells that were contaminated during isolation.

Thus, for the reasons discussed above, PARK cannot teach transdifferentiating adipocytes into cells with an osteogenic phenotype exhibiting the markers of osteogenesis.

For these reasons, PARK cannot be said to disclose each and every element of claim 15. Thus, claim 15, and all claims dependent thereon, are novel and patentable over PARK.

Thus, withdrawal of the 102(b) rejection of claims 15, 18, 23, and 24 over PARK is requested.

V. OBVIOUSNESS REJECTIONS

Claims 15-18, 23, 24, 29, and 30 were rejected under 35 U.S.C. § 103(a) as obvious over PARK taken with LECOEUR in view of SUGIHARA (Differentiation, 1986, 31:42-49) for the reasons in item 7 on pages 10-11 of the Office Action.

Claims 15-19, 23-25, and 29-32 were rejected under 35 U.S.C. § 103(a) as obvious over PARK and LECOEUR and SUGIHARA in view of ROSS (Science, 2000, 289:950-953), BENNETT (J. Biol. Chem., June 7, 2002, 277:30998-31004) and RANDO (J. Cell Biol., 1994, 125:1275-1287) for the reasons in item 8 on pages 11-12 of the Action.

These rejections are respectfully traversed and will be discussed together below in view of their common references.

It is well established that to support a *prima facie* case of obviousness, the Office must provide a rationale showing that all the claimed elements were known in the prior art and one

skilled in the art could have combined the elements as claimed by known methods with no change in their respective functions to yield predictable results. *See, KSR International Co. v. Teleflex Inc.*, 550 U.S. ___, ___, 82 U.S.P.Q.2d 1385, 1395 (2007); and M.P.E.P., Eighth Ed., Rev. 6 (September 2007) at § 2143.02.

The rejection fails, because the cited prior art references fail to disclose or suggest inducing transdifferentiation of a preadipocyte cell line obtained by dedifferentiating a mature adipocyte.

The arguments with respect to the primary reference of PARK are reiterated herein. Again, PARK fails to disclose inducing transdifferentiation of a preadipocyte cell line obtained by dedifferentiating a mature adipocyte. As discussed above, the cell used for transformation in PARK is a fibroblast derived from brown adipocyte, which was differentiated from a bone marrow stromal cell. As such, the fibroblast cell used by PARK is different from the preadipocyte in the method of independent claim 15. Also, it is believed that the cell used by PARK is not an isolated adipocyte, but a mixture of adipocyte and bone marrow stromal cell.

Again, it is respectfully submitted that the lack of clarity as to the cells actually used by PARK is such that PARK fails to teach the dedifferentiation of mature adipocytes derived from bone marrow followed by the transdifferentiation. At page

10, lines 5-6 of the Office Action, the Office acknowledges such and states "Park et al. do not teach deriving their pre-adipocytes from the dedifferentiation of mature adipocytes isolated from subcutaneous fat tissue."

Indeed, it is believed that this lack of clarity in PARK points to the unpredictable nature of the cited art teachings. As such, the rejection fails, because the cited prior art teachings could not have been combined to yield predictable results to arrive at the subject matter of claim 15.

LECOEUR and SUGIHARA fail to remedy the deficiencies of PARK.

LECOEUR was relied upon as allegedly disclosing that preadipocytes express alkaline phosphatase, which is an early maker of osteogenesis. However, such disclosure in no way describes or suggests inducing transdifferentiation of a preadipocyte cell line obtained by dedifferentiating a mature adipocyte.

As to SUGIHARA, this reference discloses a ceiling culture of adipocytes in order to obtain preadipocytes. However, SUGIHARA does not disclose preadipocytes which express an early marker, such as that of adipocyte (PPAR γ), bone cells (cbfal) and myocytes (Myf5), whereas the preadipocytes of the present claims already express those early markers. Thus, it is believed that the preadipocytes cell line of the present invention (FERM BP-0864) has unexpected properties compared to the cells of

SUGIHARA and such unexpected properties are indicative of the non-obviousness of the claims.

Even if, assuming arguendo, one of ordinary skill in the art had a reasonable motivation in doing so because ROSS teaches transforming preadipocytes to myoblasts, the skilled artisan would not have expected to transform preadipocytes into a few kinds of cells (including myoblasts) other than adipocytes. Please note the preadipocytes of the claims express not only an early marker of adipocytes and myoblast, but also an early marker of other cells. As such, even the skilled artisan would not reasonably arrive at the present invention based on the combined teachings of the references.

ROSS and BENNETT were relied upon as allegedly disclosing that adipocytes and myocytes originate from the same precursor and that signaling by Wnt10b is required for commitment to mycocyte lineage and that inhibition of Wnt10b signaling leads to adipogenesis. However, such teachings in no way describe or suggest inducing transdifferentiation of a preadipocyte cell line obtained by dedifferentiating a mature adipocyte as claim 15. Thus, ROSS and BENNETT fail to rectify the deficiencies in PARK.

RANDO was relied upon as disclosing that myoblasts grown *in vitro* can regenerate muscle fibers when transplanted into subjects. However, Applicant fails to see how such disclosure allegedly provides motivation to modify the method of PARK, LECOEUR, and SUGIHARA by transdifferentiating preadipocytes

to myoblasts as noted by the Office. Further, such teachings in no way describe or suggest inducing transdifferentiation of a preadipocyte cell line obtained by dedifferentiating a mature adipocyte as claim 15.

Therefore, Applicant respectfully submits that: (1) the cited references of PARK, LECOEUR, SUGIHARA, ROSS, BENNETT or RANDO, taken alone or in combination, do not teach, suggest or otherwise make obvious the above-noted features of claim 15; and (2) the lack of clarity in PARK signifies that the combined references would not yield predictable results to arrive at the claimed method. For these reasons, the cited references cannot render obvious independent claim 15. Thus, claim 15, and all claims dependent thereon, are novel and unobvious over the cited references.

Therefore, Applicant respectfully submits that the above-noted 103(a) obviousness rejections are untenable and should be withdrawn.

VI. CONCLUSION

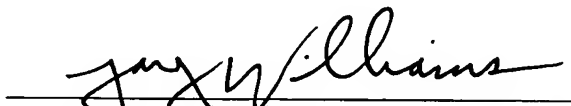
In view of the foregoing amendments and remarks, it is respectfully submitted that the present application is in condition for allowance and early notice to that effect is hereby requested.

If the Examiner has any comments or proposals for expediting prosecution, please contact the undersigned attorney at the telephone number below.

The Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 25-0120 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17.

Respectfully submitted,

YOUNG & THOMPSON



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APPENDIX:

The Appendix includes the following item(s):

- English translation of the description, claims and Abstract of JP 2000-83656.

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention]This invention relates to the establishment method of the precursor adipose cell stock of the unilocular fat cell origin of an animal.
[0002]
[Description of the Prior Art]It is shown clearly that it changes into neutral fat the mature fat cell which occupies the great portion of white adipose tissue and it not only stores the surplus energy which the living body took in, but it plays main roles in the regulation function of energy balance required for living body maintenance. For this reason, in fat cells, generation and secretion of lipid metabolism and various physiological active substances are performed actively. The diameter of the mature fat cell is called the unilocular fat cell from 10-200 micrometers and the typical gestalt which has the core by which it was pushed aside in cytoplasm at one a big lipid droplet and periphery although it is various. The morphosis of fat cells serves as a precursor adipose cell from a pluripotency mesodermal cell first, and is increased actively. Subsequently, after the commitment of the precursor adipose cell is carried out, it is supposed that a growth stop is carried out and it is changed to fat cells by an end. in this differentiation process of a series of -- fat cells -- it is known that a specific gene will be revealed tidily. research of the transcription factor (nuclear receptor) which participates in differentiation of fat cells progresses quickly these days -- fat cells -- the peroxisomal proliferation agent response receptor gamma (PPARgamma) was discovered as a master regulator who adjusts manifestation derivation and control of a specific gene cluster. PPARgamma is a nuclear receptor which is specifically revealed only to fat cells and makes with ligand fatty acid which is a nutrient them. It was also shown clearly that PPARgamma forms a retinoid X receptor and a cofactor dimer, and transcriptional control was unitedly carried out to the response arrangement (PPRE) of a target gene.
[0003]In parallel to this, PPARgamma is shown by that it is intracellular target protein of thiazolidine derivative which is a remedy to non-insulin dependent diabetes mellitus (NIDDM), and Adult diseases, such as diabetes mellitus, obesity, and hyperlipidemia, A close point of contact with research of the transcriptional control which governs adipocyte differentiation is clarified. In relevance with an adult disease, the side with the endocrine cell which generates and secretes the physiological active substance of versatility [fat cells] attracts attention. Insulin resistance is symptoms most frequently accepted in obesity and diabetes mellitus. It is thought that the insulin resistance in the diabetes mellitus accompanied by obesity is caused by TNFalpha secreted from fat cells. For Homo sapiens or the animal of obesity, TNFalpha secretion is accelerating from the fat cells from visceral fat, and correlating with the index of insulin resistance is actually shown. PAI-1 (plasminogen activator inhibitor 1) is the most important substance in a blood fibrinolysis system, fibrinogenolysis nature is reduced, thrombosis is promoted and becoming causes, such as myocardial infarction, is known. Although PAI-1 in blood goes up in obesity and a NIDDM patient, it is also shown clearly that they are mainly of visceral fat cell origin. Although it is new hormone which the leptin which is a product of an obesity gene is produced by fat cells, acts on a center, promotes food intake control and energy expenditure,

and adjusts body fat uniformly, This is also high in obesity and a NIDDM patient, and it is also shown clearly that production rises by TNFalpha. Since the rise of TNFalpha [in obesity or the blood in NIDDM] and PAI-1 and leptin improves strongly by thiazolidine derivative, it is thought that generation and secretion of the physiological active substance of these fat cell origin are directly related to adipocyte differentiation. However, many points the knowledge of whose in the explantation which used the mouse precursor adipose cell stock about the mechanism of adipocyte differentiation in Homo sapiens the knowledge about a disease is most and is still unknown about the relevance of those diseases and adipocyte differentiation are left behind.
[0004]On the other hand, control of body fat accumulation of industrial animals, such as livestock or domestic fowls, and creation of fat cross meat were performed by regulation of feed energy or a nutrient until now, and came. However, as a breeding objective of economic effect priority, since priority was given to improvement in gain of body weight, the individual with many amounts of feed intake has been selected inevitably. There are many individuals which have a tendency which falls into hyperenergia easily by an individual with many amounts of feed intake by that cause, and is accumulated as a fat. In contrast, in Homo sapiens, low-fat meat is liked from the increase in obesity, and the actual condition is discarded without eating the fat of most which is produced. Simultaneously, the increase in the illness by the cacochymia which originates in superfluous accumulation of body fat also in livestock or domestic fowls has also been a problem. It seems that controlling superfluous accumulation of body fat has already reached the limit, maintaining high productivity by the conventional indirect method as these solution. Then, if the growth and the differentiation mechanism of fat cells which constitute fat tissue are directly controllable as a fundamental theme, it will be thought that more effective control of body fat accumulation is enabled. However, the experiment system whose actual condition is that most accumulation of the knowledge in the cell level about the growth and differentiation of fat cells which constitute the fat tissue of livestock or domestic fowls is not made and which excelled for investigating it is not yet established, either.
[0005]Until now the research on growth and differentiation of fat cells, It has been carried out using the primary culture system of the precursor adipose cell (S-V cell) mainly contained in the precursor adipose cell stock of Swiss-3 T3 origin (3 T3-L1 and 3 T3-F443A), or the stromata-blood vessel fraction obtained by carrying out enzyme treatment of the fat tissue. However, in the precursor adipose cell stock of Swiss-3 T3 origin. 1) the germ on 17 to 2 pregnancy the 19th which the variant cell with the chromosome from which the pattern differed is mixing, since it is the origin and differentiation inducing will be spontaneously carried out, if it cultivates by three serum containing media in which the differentiation characteristic differs from the precursor adipose cell of adult origin, The issue which cannot specify the substance which carries out differentiation inducing intrinsically and which should solve ** occurs. Since cells other than 1 precursor adipose cell, for example, a vascular endothelial cell, a smooth muscle cell, fibroblast, etc. are mixing the S-V cell on the other hand, In investigating the differentiation characteristic of the precursor adipose cell itself, or 2 fat tissue which are not made (influence of other cell populations cannot be disregarded), Since it is three primary culture systems which a commitment is already carried out and result in the cell on the way of differentiation to fat cells from the cell which just specialized from the multipotential cell of mesoderm nature to the precursor adipose cell and in which the cell in the differentiation process of various stages exists, the experiment of multiple times cannot be performed using an identical material — 4 — since the difference between the individuals which prepare a cell from said reason of 2 and 3 is large, the high data of reproducibility is not obtained — the issue which should solve ** occurs.
[0006]Although these technical problems may carry out cloning of the precursor adipose cell of the target animal by limiting dilution etc. and it may be able to solve by establishing a precursor adipose cell stock, such operation is complicated and its success percentage is low.
[0007]
[Problem(s) to be Solved by the Invention]Then, this invention makes it a technical problem to provide the precursor adipose cell stock obtained by the new establishment method and this method of a precursor adipose cell of the unilocular fat cell origin of an animal.
[0008]

[Means for Solving the Problem]As a result of inquiring wholeheartedly that an aforementioned problem should be solved, this invention persons already, Fibroblast Mr. fat cells which have accomplished end differentiation and which are obtained by carrying out ceiling culture of the unilocular fat cell of animal origin, It checked having the same growth as a precursor adipose cell, and the differentiation characteristic, further, even if it carried out subculture of this fibroblast Mr. fat cell for a long period of time, it found out that held uniform growth and differentiation potency and subculture was made without a transformation, and this invention was completed. Namely, this invention carries out subculture of the fibroblast Mr. fat cells formed by carrying out ceiling culture of the unilocular fat cell of one animal, It is related by carrying out differentiation inducing, without a precursor adipose cell stock of this animal origin acquired, a precursor adipose cell stock given in 1 whose two animals are Homo sapiens, a precursor adipose cell stock given in 1 whose three animals are swine, and a precursor adipose cell stock given in 1 whose four animals are swine.

[0009]

[Embodiment of the Invention]It is shown clearly that the fat tissue of an animal has the characteristic which is different to every [which is formed] parts (inside of a mesentery, the kidney circumference, hypodermic, an epididymis, and the muscular system, etc.). Then, age or sex of an animal, etc. can choose the fat tissue to extract for the purpose of establishing a precursor adipose cell. For example, it aims at high meat (for example, marbled meat) production of commodity value in livestock, It aims at improvement in the rate of a carcass of establishment of the precursor adipose cell stock of the muscular system origin for a molding machine style break through of the fat cells in the muscular system, the livestock for meat, or domestic fowls, In establishment of the precursor adipose cell stock of the intraperitoneal fat origin for the molding machine style of intraperitoneal fat cells, and its characteristic break through, establishment of the precursor adipose cell stock of the mammary gland organization origin aiming at investigation of the relation of milk fat and fatty acid composition, or Homo sapiens, Establishment of the precursor adipose cell stock of the visceral fat tissue origin for generation of the physiological active substances (TNFalpha, leptin, PAI-1, etc.) which participate especially in obesity or an adult disease deeply, and a regulatory mechanism break through of secretion etc. can be considered.

[0010]1) Fundamentally, separation of the separation unilocular fat cell of a unilocular fat cell can be performed according to the method (RodbelM.:J.Biol.Chem., 239:173-181-1964) of Rodbel. The fat tissue made into the purpose from Homo sapiens, or the livestock and domestic fowls under surgical operation is extracted quickly. A thicker blood vessel and connective tissue are removed. Fat tissue is put into the basal medium (for example, Dulbecco's modified Eagle's medium) containing an antibiotic or phosphate buffered saline, and fat tissue is washed gently. Subsequently, fat tissue is put in the culture medium containing enzymes, such as bovine serum albumin and collagenase, trypsin, pronase, DISUPA, elastase, and hyaluronidase, after carrying out a fragment so that a cell may not carry out the detrition with the scissors for surgery, it shakes for 45 to 60 minutes, and a cell is distributed. After distributed operation, cell suspension is filtered by a nylon mesh and an undigested organization is removed. If filtrate is centrifuged gently, unilocular fat cells will float and gather for the upper layer. On the other hand, stromata vascular cells (a precursor adipose cell is included) gather for precipitate. Suction extraction of the unilocular fat cell is carried out by BIPETTO. Centrifugal washing of the extracted unilocular fat cell is further moved and carried out to a culture medium (for example, Dulbecco's modified Eagle's medium containing a blood serum or BSA) several times.

[0011]2) Culture of the culture unilocular fat cell of a unilocular fat cell can be performed according to the method (Sugihara, H.et al.:Differentiation, 31:42-49-1986) of Sugihara and others. That is, although a unilocular fat cell floats in culture medium with the neutral fat contained in cytoplasm, it is the method (ceiling culture) of making paste up this cell on the inner side upper surface (ceiling surface) of the flask made filled with a culture medium 100%, using floating [this] conversely, and cultivating. If ceiling culture of the unilocular fat cell is carried out for several days by this method, some cytoplasm will be made to elongate or extend, a flask ceiling surface will be pasted firmly, and the shape change of most cells will be carried out to the

multilocular fat cells which have a lipid droplet of various sizes around a large-sized lipid droplet. At this time, the culture medium in a flask is exchanged for optimum dose of culture media, it returns to the usual cultivation, and culture is continued. The lipid droplet contained in multilocular fat cells is divided further, multilocular fat cells expand cytoplasm further and the cell which changes to the gestalt like fibroblast is observed as it becomes small. If culture is furthermore continued, a majority of fibroblast Mr. fat cells (FA) which slightly or completely do not have a lipid droplet will come to be observed by the periphery of the multilocular fat cells which present the gestalt like fibroblast. While increasing FA actively, multilocular fat cells are no longer observed gradually. Then, the cell in a flask serves as only FA and reaches confluence. When ceiling culture of the unilocular fat cell extracted from which kind is carried out, after [which was shown above] carrying out a shape change, it increases actively, but a swine, a fowl, a rat, or Homo sapiens changes a little with parts (for example, hypodermic or visceral fat tissue), sex, etc. of age and the organization which extracted.

[0012]3) Perform the extraction and subculture of FA which were formed in growth of fibroblast Mr. fat cells (FA), and the differentiation above 2 using the method shown below. FA being formed after ceiling culture and in a flask, and removing the culture medium in a flask, if increasing actively is checked -- the cell in a flask — trypsinization -- and it centrifuges. FA which does not have a lipid droplet for the multilocular fat cells which have a lipid droplet in the upper fraction by this operation is separable into a precipitate fraction. If subculture of the FA of this unilocular fat cell origin is carried out, while almost all cells are pasted up on the culture dish bottom and the gestalt like fibroblast is shown 24 hours after culture, it will increase actively, and will reach almost confluent in several days. The growth character of FA of this animal or organization origin is investigated by creating the proliferation profile in this incubation period. Although a lipid droplet minute in cytoplasm is observed depending on animal species (Homo sapiens or rat), a dedifferentiation derivation operation of fat cells is suitably performed using the culture medium which it has and which carried out TNFa addition etc. in this case. The check of the existence of the lipid droplet in cytoplasm is performed using oil red 0 staining technique. A lipid droplet is not observed in FA of a swine or a fowl.

[0013]About the differentiation characteristic of FA, it carries out using the method shown below. The culture medium of FA is exchanged for the culture medium (it differs a little by animal species) which added the differentiation inducer, culture culture is carried out for several days, and differentiation inducing is performed. It returns to the usual culture medium after differentiation inducing, and culture is continued further. Generally, if several days will go through FA after differentiation inducing, the shape change of the FA will be carried out to the shape of a star, or a valvate form, and the cell which has a small lipid droplet in cytoplasm will come to be observed. If culture is furthermore continued, cytoplasm will be extended and the lipid droplet of various sizes will come to be observed in cytoplasm. The check of the lipid droplet accumulation in FA cytoplasm after differentiation inducing is performed using oil red 0 staining technique. The glycerol-3-phosphate-dehydrogenase (GPDH) activity which is a second-half marker of differentiation of fat cells is measured as an index of differentiation, and change of the activity in the incubation period after differentiation inducing is observed. If it is shown that this animal or FA of an organization has active growth and differentiation potency as the above result, it is shown that FA is a precursor adipose cell of unilocular fat cell origin.

[0014]4) Perform subculture of the precursor adipose cell of the establishment unilocular fat cell origin of a precursor adipose cell stock, and try establishment of a precursor adipose cell stock by investigating the growth and differentiation potency for every passage. For example, in the precursor adipose cell of the unilocular fat cell origin of a swine and a fowl, it is checked that the same uniform proliferation potential power and differentiation potency as the early stages of a passage are maintained also in 37 and the 33rd generation, respectively, and the transformation of a chromosomal aberration etc. is not observed, either.

[0015]As mentioned above, this invention consists of about four lines. Although this invention showed establishment of a swine subcutaneous adipose tissue and the precursor adipose cell stock of fowl intraperitoneal fat tissue origin below as an example, he is a person skilled in the art, using other livestock, for example, a cow and a sheep, or the duck of domestic fowls, a quail,

and the art further indicated by Homo sapiens etc. by this invention — if it becomes — a required change — in addition, it is possible to establish the target animal and the precursor adipose cell stock of organization origin. For example, if selection of the culture medium on a culture condition, serum concentration, a differentiation inducer, etc. is a person skilled in the art, it can set up an optimal condition suitably as a result of easy trial and error. For example, in the case of the precursor adipose cell of 14 age-in-day fowl abdominal fat cell origin. Blood serum addition addition concentration uses 10%, and a differentiation inducer uses fatty acid, and, in the case of the precursor adipose cell of six to seven-month age swine subcutaneous adipose tissue origin, the simultaneous adding of an insulin, dexamethasone, and isobutylmethylxanthine is suitable for a differentiation inducer at 20% blood serum addition.

[0016]

[Example]Hereafter, although an example explains this invention concretely, this invention is not limited to these examples.

[0017][Example 1] It carried out in order to clarify the growth character of the growth character swine unilocular fat cell of a unilocular fat cell. That is, the unilocular fat cell was cultured using ceiling cultivation, and the growth form was observed. Subsequently, it was also examined whether those efficient extraction would be possible by investigating the proliferation state of FA formed from a unilocular fat cell.

[0018]1) At material, and extraction and the ** place of method (1) fat tissue, the male of 6 - seven-month age or the subcutaneous adipose tissue of the sow was extracted, and it put into the incubation bottle adjusted to about 37 **, and brought back to the laboratory within 1 hour.

[0019](2) Isolation of a unilocular fat cell, isolation of a culture unilocular fat cell, and the outline of a culturing method are shown in drawing 1. The solution which added Sept Iles trimethylammonium bromide 0.1% (v/v) to the phosphate-buffered saline (PBS-PVA) of 0.08mg [/ml] kanamycin sulfate (SIGMA) and 0.5mg [/ml] poly vinyl alcohol (SIGMA) addition washed fat tissue. The organization was washed 3 times by PBS-PVA. To the Dulbecco's modified Eagle's medium (HEPES-DMEM; NISSUI PHARMACEUTICAL) which added 25mM HEPES, 1.8 mg/ml $NaHCO_3$, and 0.08mg [/ml] kanamycin sulfate. About 4 g of fat tissue was moved into the culture medium (pH 7.4) which added bovine serum albumin (BSA) and 0.1% collagenase (Type II;SIGMA) 2 more%, and the fragment was carried out using the scissors for surgery. Subsequently, after moving to a centrifugation tube, shaking culture was carried out for 60 minutes within a 39 ** culture apparatus. After filtering cell suspension after collagenase treatment using the caliber 250 and a 150-micrometer nylon mesh (**** science and engineering), The cell which adhered to a mesh in HEPES-DMEM which added the cow fetus blood serum (FCS;Filtron) 3% (v/v) was flushed, and undigested tissue and a cell were classified. Cell suspension was moved to the centrifugation tube and the fraction which becomes the upper layer from a unilocular fat cell was obtained for 3 minutes by ** centrifuged by 106G. Suction extraction of the unilocular fat cell fraction was carried out with the pipette, and it was moved into fresh 3%FCS addition HEPES-DMEM. After repeating centrifugal washing for [106] G or 3 minutes 3 times, the cell number was measured using the erythrocytometer.

[0020]Culture of the unilocular fat cell was performed according to the method of Sugihara and others (Sugihara, H.etal.:Differentiation, 31:42-49-1986). That is, the unilocular fat cell of the 3 - $6x10^5$ individual was moved to the tissue culture flask (Falcon, 3107), and the inside of a flask was thoroughly filled with DMEM which added FCS, 1.8 mg/ml$NaHCO_3$, and 0.08mg [/ml] kanamycin sulfate 20%. Into the CO'2 incubation device under the gaseous phase of 37 **, 5% CO_2, and 95% air, it settled so that the flask bottom might become a top, and it cultivated for six days. After removing the culture medium six days after culture and in a flask, it exchanged for the FCS addition DMEM 20%, and as the cell adhesion side turned into the bottom, it continued culture for 16 more days within the CO'2 incubation device. Culture-medium exchange after ceiling culture was performed day by day [4]. About the proliferation state of the unilocular fat cell, it observed under the inverted microscope every day.

[0021](3) FA increased from the unilocular fat cell was measured by the method shown below

the measurement culture 6, 10, 14, and 18 and 22 days after the number of FA. After removing the culture medium in a flask, it washed 4 times by PBS-PVA. Subsequently, after adding in a flask PBS (trypsin EDTA-PBS) which added trypsin (Difco) and 0.1% (w/v) ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque) 0.1% (w/v), it settled for 5 minutes into the CO'2 incubation device. After culture, after adding the FCS addition DMEM 3%, it moved to the centrifugation tube, and the inside of a flask was further washed twice by the culture medium, and cells were collected. Then, after carrying out at-long-intervals heart washing for 165 G or 3 minutes and making a cell re-float by the FCS addition DMEM 20%, the cell number was calculated using the erythrocytometer.

[0022](4) Oil red 0 dyeing (Hausman, G.J.:Stain Technology, 56:149-154-1981) was used for histochemical search of the adiposity in histochemical search cytoplasm. That is, formalin (v/v) addition PBS (formalin PBS) was added to the culture medium in a flask 10%, and it front-fixed under the room temperature for 20 minutes. The culture medium in a flask was removed, formalin PBS was added 10% again, and it back-fixed under a 1-hour room temperature. Then, formalin PBS in a flask was removed and distilled water washed 2 to 3 times. After mixing distilled water with a 0.5% (w/w) oil red 0-isopropyl alcohol solution by 3:2, oil red 0 stain solution filtered and created with qualitative filter paper.(No.2, Advantec) was put into the flask, and it dyed under a 1-hour room temperature. Distilled water washed 2 to 3 times after dyeing, and after making it air-dry, unilocularity, multilocular, and the adiposity situation of FA were observed under the inverted microscope.

[0023]2) Join By the centrifugation operation after ** collagenase treatment, the single cell fraction which consists of unilocular fat cells was obtained (drawing 2 - a). The number of unilocular fat cells extracted from the subcutaneous adipose tissue was an abbreviation $3x10^6$ individual per 4g.

[0024]All the unilocular fat cells introduced in the flask came floating to the flask ceiling surface (bottom), and presented the typical gestalt which has the core which was extruded by one big lipid droplet contained in cytoplasm, and moved to the periphery of the cell. Two to three days after ceiling culture, although some unilocular fat cells were imperfect, the flask ceiling surface was pasted. Four days after culture, some cytoplasm was made to elongate or extend, the flask ceiling surface was pasted firmly, and the shape change of most cells was carried out to the multilocular fat cells which have a lipid droplet of various sizes around a large-sized lipid droplet. The lipid droplet contained in multilocular fat cells was divided further, multilocular fat cells expanded cytoplasm further and the cell which changes to the gestalt like fibroblast was observed as it became small. Six days after culture, much FA which does not have a lipid droplet at all was observed by the periphery of the multilocular fat cells which present the gestalt like fibroblast (drawing 2 - a). Then, although FA without a lipid droplet was increased actively, multilocular fat cells are no longer observed gradually (drawing 2 - b). The cell 14 days after culture and in a flask was set only to FA, and reached confluence (drawing 2 - c).

[0025]It was able to do [dividing into a precipitate fraction FA which is not in the upper fraction about a lipid droplet in the multilocular fat cells which have a lipid droplet for the cell in a flask trypsinization and by centrifuging, either, or] after the end of ceiling culture (six days after culture). The proliferation profile of FA after ceiling culture was shown in drawing 3. FA extracted six days after culture -- about 6 -- after [of culture] 18 days which were increased quickly after that and attained confluent although it was a $x10^4$ individual / $25cm_2$ -- about 1.4 -- it increased even by a $x10^6$ individual / $25cm_2$, and about 23 times. Increasing it actively to multilocular fat cells, after carrying out the shape change of the swine unilocular fat cell to FA further, and reaching even confluence from the above result, was shown. Therefore, when carrying out ceiling culture of the swine unilocular fat cell, it became clear that FA which is a useful material for precursor adipose cell stock establishment is simple and efficiently extractable.

[0026][Example 2] When ceiling culture of growth of swine FA and the differentiation characteristic swine unilocular fat cell was carried out, Example 1 showed that efficient

extraction of FA which does not have a lipid droplet in cytoplasm was possible. If extracted FA has active proliferation potential and the redifferentiation ability to fat cells after subculture, those cells are precursor adipose cells. In this example, it carried out in order to investigate whether FA obtained from the unilocular fat cell dedifferentiates even to a precursor adipose cell, i.e., is FA a precursor adipose cell?. First, FA cell made the control plot the S-V cell which extracted from fat tissue whether it would have the same growth as a precursor adipose cell, and the differentiation characteristic, and carried out the comparative examination. It investigated also about the optimal conditions in growth and differentiation of FA.

[0027]1) Extraction of material and growth character FA of method (1) FA was performed by the same method as (1) of Example 1. After removing the culture medium 14 days after ceiling culture and in a flask, it washed 4 times by PBS-PVA. After adding trypsin EDTA-PBS in a flask, it settled for 5 to 8 minutes into the CO'2 incubation device. After culture, after adding the FCS addition DMEM 3%, it moved to the centrifugation tube, and the inside of a flask was further washed twice by the culture medium, and cells were collected. Then, after carrying out at-long-intervals heart washing for 165 G or 3 minutes and making a cell re-float by the FCS addition DMEM 20%, the cell number was measured using the erythrocytometer.

[0028]On the other hand, extraction of the S-V cell which is a control plot carried out cell suspension after centrifugality, and carried out suction removal of the upper fraction with the pipette, and it carried out like (2) of Example 1 except having extracted the S-V fraction of settling.

[0029]After carrying out seeding of FA and the S-V cell to each of DMEM which added FCS and 20%FCS 10% so that it may be set to 10^4, and 10^5 individual / ml, seeding was carried out to a 35-mm culture dish (FAlcon, 3001J). It settled into the 5%CO_2 CO'2 incubation device, and each cell number was culture measured day by day [4] to the backward one on the 16th. Observation of the proliferation state of FA and a S-V cell was performed every day using the inverted microscope.

[0030](2) Differentiation characteristic FA or the S-V cell of FA was adjusted by the FCS addition DMEM 20%, seeding was carried out to the culture dish so that it might be set to a last concentration $1x10^4$ individual / ml, and it was cultured for ten days within the CO'2 incubation device (37 **, 5%CO_2, 95% air). After culture and after checking a confluent state, they are various concentration [FCS], Dexamethasone (DEX;0-2.5microM), a 1-methyl-3-isobutylxanthin (IBMX;0 - 5mM), Or about differentiation inducers, such as an insulin (INS; 0-50 micro, g/ml), it exchanged for the differentiation-inducing culture medium added in various concentration or combination, and cultivated for four days. After culture, it exchanged for the FCS addition DMEM 20% again, and cultivated for eight more days. The differentiation situation investigated glycerol 3 phosphoric-acid dehydrogenase (G3 PDH) specific activity value and oil red 0 dyeing as an index.

[0031]Adjustment of the cell used for G3PDH activity measurement was performed according to the method of Pairault and Green (Pairault, J.and Green, H.-roc.Natl.Acad.Sci.USA, 76:5138-5142-1979). That is, the culture medium of the culture dish was removed 12 days after day by day [4] or culture until after the culture 12, after washing a cell twice by PBS beforehand cooled at 4 **, 25 mMTris-HCl (pH 7.5) which carried out 1mM EDTA addition was added, and the cell was gathered up by the rubber policeman. Tris-HCl including a cell lump was moved to the micro tube, ultrasonic crushing treatment was carried out for [150] W or 10 seconds, and the cell was destroyed. Subsequently, after centrifuging for 5 minutes by 4 ** and 12800G, supernatant liquid was moved to the tube for ultracentrifuges, and was centrifuged for 100000 G or 60 minutes 4 **. The supernatant liquid (crude enzyme liquid) obtained after centrifugality was moved to the micro tube, and it saved at -80 ** just before G3PDH activity measurement.

[0032]Measurement of G3PDH activity followed the method of Kozak and Jensen (Kozak, L.P.and Jensen, J.T.:J.Biol.Chem., 249:7775-7781-1974). That is, what mixed the mixed solution 50mu1 of 0.5H triethanolamine, 10mM EDTA, and 10mMbeta-mercaptoethanol, 5mM dihydroxyacetone phosphate (SIGMA), and 0.5mM NADH (oriental yeast) was used as reaction mixture. Immediately

after adding the crude enzyme liquid immediately after fusion to reaction mixture and agitating it, change of the absorbance per unit time was measured using the spectrophotometer (25 **, 340 nm).

[0033]The protein concentration in crude enzyme liquid was measured in accordance with the method of Lowry and others. That is, what was mixed so that 1%(w/v) $CuSO_4$, 2%(w/v) Na_2CO_3 and $5H_2O$, and 2% (w/v) tartaric acid calina thorium might be set to 50:1 in the crude enzyme liquid diluted with distilled water 25 times was added, and it settled under the room temperature for 10 minutes. Then, after adding IN phenol reagent, it settled for 30 minutes. The absorbance was measured after the coloring reaction using the spectrophotometer (750 nm). The G3PDH specific activity value (units/mgprotein) was computed using the expression shown below from the G3PDH activity value and protein content which were obtained by the above operation.

[0034]G3PDH specific activity value (units/mg protein) = (the amount of absorbance variations / 1.25x t minutes for 100xt minutes) protein concentration in /crude enzyme liquid (mg)

2) Join The shape change in the proliferation period of FA in which ** (1) FA carried out growth character subculture was investigated. Almost all the cells by which seeding was carried out were pasted up on the culture dish bottom irrespective of FA and a S-V cell 24 hours after culture. Then, it elongated, and FA and the S-V cell which were pasted up showed the gestalt like fibroblast, and increased it actively. Ten days after culture, any cell spread on the culture dish whole surface, and was attained almost confluent. The morphological difference between FA in a proliferation period and a S-V cell was not observed. The proliferation profile of FA and a S-V cell was shown in drawing 4. In 10^5 individual / ml seeding division of FA and a S-V cell, irrespective of FCS concentration, it increased quickly and reached confluent eight days after culture. On the other hand, also in 10^4 individual / ml seeding division, irrespective of FCS concentration, FA and a S-V cell increased quickly, and were attained almost confluent 12 days after culture. However, by the 20%FCS additive area of FA, the increase in a cell number was accepted 12 days after culture, and the same value as 10^5 individual / ml seeding division was shown 16 days after culture.

[0035](2) The shape change accompanying differentiation of differentiation characteristic FA of FA was shown in drawing 5. Even if it attained FA in front of differentiation inducing confluent, the typical gestalt like fibroblast was shown and the lipid droplet was not observed in cytoplasm (drawing 5 - a, b). When differentiation inducing was carried out using DMEM which added 0.25microM DEX, 5microg/ml INS, 0.5mM IBMX, and 20%FCS, four days after differentiation inducing, FA showed the star-like gestalt and what has a small lipid droplet in cytoplasm was observed (drawing 5 - b). 12 days after differentiation inducing, cytoplasm was extended and many lipid droplets of various sizes were observed in cytoplasm (drawing 5 - c). Although were not shown in a figure, and the part became star-like also in the S-V cell after differentiation inducing, almost all other cells maintained the gestalt like fibroblast. However, 12 days after culture, cytoplasm was extended like FA, and the cell which accumulated the lipid droplet of various sizes into cytoplasm was also observed.

[0036]Even if it dyed FA which reached the confluence in front of differentiation inducing oil red 0, the dyed cell was not observed at all (drawing 5 - d). However, dyeing [oil red 0] of FA 12 days after differentiation inducing showed that most FA at the bottom of a culture dish which the whole was dyed oil red 0 (drawing 5 - f), and cultivated mostly specialized to a mature fat cell. On the other hand, although the portion dyed oil red 0 in the shape of a colony was observed in the S-V cell, there were few grades of dyeing compared with FA (drawing 5 - g). As for FA which does not carry out differentiation inducing, the gestalt like fibroblast was maintained in all the incubation period, and most positive cells were not observed by oil red 0 dyeing 12 days after culture (drawing 5 - e).

[0037]Change of the G3PDH specific activity value after differentiation inducing of FA and a S-V cell was shown in drawing 6. In a differentiation inducer additive-free division, the rise of the G3PDH specific activity value was not accepted to the backward one in any of FA and a S-V cell on culture the 12th. This result shows that differentiation inducing of the FA is carried out

and it does not cause spontaneous differentiation only with a differentiation inducer. On the other hand, the G3PDH specific activity value of FA in a differentiation inducer additive area and a S-V cell rose quickly after the differentiation inducing 4 and eight days, respectively, and the difference between each culture days was significant. The G3PDH specific activity value of FA 12 days after differentiation inducing and a S-V cell is 54units [160 and]/mg protein, respectively.
The G3PDH specific activity value of FA showed the value about 3 times higher compared with the S-V cell.

[0038]The influence of serum concentration on differentiation of FA was investigated. Differentiation inducing was carried out to 0.25microM DEX, and 5microg/DMEM which carried out ml INS and 0.5mM IBMX addition by 5, 10, 20, or the culture medium that carried out FCS addition 40% (v/v), and it cultivated for 12 days. As a result, the G3PDH specific activity value increased to the concentration dependence target between 5 to 20%FCS additive areas, and the significant difference was accepted at each section. However, in the 40%FCS additive area, the tendency for a G3PDH specific activity value to fall was accepted. From these results, it was shown in differentiation inducing of FA that the 20%FCS addition to a differentiation-inducing culture medium is optimum concentration.
[0039]The influence which the combination of differentiation inducers, such as DEX and IBMX which are added to a culture medium, and INS, has on differentiation of FA was investigated (drawing 7). three sorts of differentiation inducers -- it was independent respectively, or it combined and added and the G3PDH specific activity value 12 days after culture was measured. As a result, the G3PDH specific activity value of IBMX and DEX showed the high value intentionally compared with the INS additive area and the control plot. When two sorts of differentiation inducers were combined, in all the divisions, the high value was intentionally shown compared with the control plot and the INS division. In particular, the synergistic effect was accepted and the DEX+IBMX division showed the high value intentionally compared with an INS+IBMX division and all the independent additive areas. The specific activity value of the DEX+IBMX+INS division which combined three sorts showed 132units/mg protein and a remarkable synergistic effect, and showed the high value intentionally ($p< 0.001$) compared with a control plot and all the experiment divisions. Subsequently, the optimum concentration of each differentiation inducer contained in a DEX+IBMX+INS division was investigated. As a result, although it also set to any of DEX, IBMX, and INS and the G3PDH specific activity value 12 days after culture was raised on the concentration dependence target to 0.25microM, 0.5mM, and 5micro/ml, respectively, change was not accepted by the concentration beyond it.
[0040]FA of unilocular fat cell origin became clear [that it is a precursor adipose cell which has active growth and differentiation potency] from the above result.
[0041][Example 3] In the influence example 2 of the number of times of a passage exerted on growth and differentiation of a swine precursor adipose cell, FA formed from a unilocular fat cell became clear [that it is a precursor adipose cell]. Therefore, a passage is possible for the precursor adipose cell of this unilocular fat cell origin (Porcine Peradipocytes derived from Matured Adipocytes:PPMA), If growth and differentiation potency stable even if it repeated the passage again are maintained, it will come out of PPMA in ** and others that it is a swine precursor adipose cell stock. In this example, subculture of the PPHA was carried out over the long period of time for the purpose of establishment of a swine precursor adipose cell stock, and the comparative examination of the growth and differentiation potency between passages was carried out.
[0042]1) in material, and extraction and the ** place of the precursor adipose cell of method (1) swine unilocular fat cell origin -- male [of 6 - seven month age], or sow type 5 -- the subcutaneous adipose tissue was extracted flatly, and it put into the incubation bottle adjusted to about 37 **, and brought back to the laboratory within 1 hour. Subsequently, ceiling culture was carried out by the method of Example 1, and PPMA was obtained.
[0043](2) The subculture of a swine precursor adipose cell and the subculture method of the differentiation inducing PPMA were performed according to the preceding clause. That is, after

removing PPMA from the culture dish bottom and carrying out centrifugal washing using trypsin EDTA-PBS, the cell number was computed using the erythrocytometer. Subsequently, it was made to re-float by the FCS addition DMEM 20% so that it may be set to a last concentration 1×10^4 individual / ml, 2 ml of cell suspension was moved to the culture dish, and it cultivated for ten days within the CO'2 incubation device under the gaseous phase of 37 **, 5%CO_2, and 95% air. Culture-medium exchange was performed day by day [4], and the above operation was repeated for every passage. About observation of the proliferation state, it observed every day using the inverted microscope.

[0044]About differentiation inducing, differentiation inducing of the culture medium of PPMA attained confluent ten days after subculture was exchanged and carried out to the 20%FCS addition DMEM containing 0.25microM DEX, 0.5mM IBMX, and 5microg/mlINS for four days. Then, culture-medium exchange was carried out 20% at the FCS addition DMEM, and it cultivated for 5 and six more days. PPMA was sampled ten days after culture and the G3PDH specific activity value was measured in accordance with the method shown in the preceding clause 2. Observation of the cell form change after differentiation inducing was performed every day using the inverted microscope.

[0045]2) Join PPMA in ******** showed the gestalt like fibroblast, and the morphological difference between each passage was not observed. The influence of the number of times of a passage exerted on growth of PPMA was shown in drawing 8-a. By PPMA of four individual, reduction of the cell number ten days after culture was accepted in the 6-7th generation of the passage among five individuals. However, it was stabilized after that and the same proliferation potential power as the early stages of a passage was maintained also in the 37th generation of the passage in PPMA of 3 individual origin.

[0046]Although not shown in a figure, the chromosome number of PPMA in the 35th generation of the passage was calculated by analysis of the metaphase image. 74% of PPMA was diplont (38 chromosomes), and 11% was 39 chromosomes. 8% was 38 or less chromosomes, and they were 58 or more chromosomes a structural anomaly and 1% of 6%. After a long-term passage culture in PPMA, normal phenotype is maintained and these results are ******(ed).

[0047]As for the shape change observed after differentiation inducing of PPMA, the difference was not observed between each passage. In PPMA of 3 individual origin, even if it repeated the passage of 35 or more generation, the lipid droplet of various sizes was accumulated into cytoplasm, and the morphological difference with the early stages of a passage was not accepted. The influence of the number of times of a passage exerted on differentiation of PPMA was shown in drawing 8-b. In PPMA of 3 individual origin, the G3PDH specific activity value was maintained to the 37th generation of the passage. However, the G3PDH specific activity value of PPMA of other 2 individual origin, It decreased quickly from the passage 3 and the 16th generation, respectively, and each of passages 12 and G3PDH specific activity values after the 22nd generation fell to the same value as the control plot (2 - 6units/mg protein) which does not carry out differentiation inducing, respectively. Even if PPMA of unilocular fat cell origin passes through long-term passage, the above result shows that uniform growth and the differentiation characteristic are maintained, and requires it. Therefore, PPMA became clear [that it is a swine precursor adipose cell stock in which a long-term passage culture is possible].

[0048][Example 4] In differentiation characteristic this example of fowl FA, it investigated about whether FA obtained by carrying out ceiling culture of the unilocular fat cell extracted from the abdominal fat tissue of the fowl dedifferentiates even to a precursor adipose cell. The S-V cell extracted from intraperitoneal fat tissue was made into the control plot.

[0049]1) After killing material and a method 14 age-in-day male fowl with bleeding, the intraperitoneal fat was extracted promptly and weight was measured. Extraction of a unilocular fat cell and FA and those cultivation were performed by the almost same method as (1) of Example 1. On the other hand, extraction of the S-V cell was performed by the same method as (1) of Example 2. In the case of the fowl, what carried out subculture of the FA formed eight days after ceiling culture was used. FA or a S-V cell was adjusted by the FCS addition DMEM 10%, seeding was carried out to the culture dish so that it might be set to a last concentration

1x10 ^{5} individual / ml, and it was settled into the CO'2 incubation device (37 **, 5%CO_2, 95% air), and was cultured for eight days. After culture and after checking a confluent state, to DMEM containing a 1 microg [/ml] insulin, 10 microg [/ml] transferrin, and 12 mg/ml BSA, it exchanged for fatty acid concentrate (GIBCO BRL) additive-free or the added culture medium 0.1%, and cultivated for 12 days. Culture-medium exchange was performed day by day [4]. The differentiation situation investigated G3PDH specific activity value and oil red 0 dyeing as an index. The method of adjustment of a cell, G3PDH specific activity measurement, and oil red 0 dyeing used for G3PDH activity measurement followed (2) of Example 2.

[0050]2) Join Change of the G3PDH specific activity value after differentiation inducing of ** fowl FA and a S-V cell is shown in drawing 9. Although the tendency for the G3PDH specific activity value four days after culture to rise a little also in any of FA and a S-V cell was accepted in the fatty acid additive-free division, after that, it fell [culture] to the backward one on the 12th, and the significant rise of the G3PDH specific activity value was not accepted. in the fatty acid additive-free division, the substance of the oil red 0 dyeing positivity was not observed by cytoplasm in any of incubation period. On the other hand, although the G3PDH specific activity value of FA four days after [of differentiation inducing] in a fatty acid additive area and a S-V cell is the same as that of an additive-free division and the significant rise was not accepted, Each of FA eight days after differentiation inducing and G3PDH specific activity values of the S-V cell rose quickly, and the specific activity value of FA showed the high value intentionally compared with the S-V cell. The G3PDH specific activity value 12 days after differentiation inducing all indicated the peak prices to be 79 and 60 units/protein, respectively. From these results, it was shown that fowl FA is a precursor adipose cell. Differentiation inducing of them was carried out with fatty acid, and not causing spontaneous differentiation was shown.

[0051][Example 5] In the influence example 4 of the number of times of a passage exerted on growth and differentiation of a fowl precursor adipose cell, it became clear that FA formed from a fowl unilocular fat cell is a precursor adipose cell. A passage is possible for the precursor adipose cell of this unilocular fat cell origin (Chick Preadipocyte drived from MaturedAdipocytes:CPMA), If growth and differentiation potency stable even if it repeated the passage are maintained, it is thought that CPMA is a fowl precursor adipose cell stock. In this example, subculture of the CPMA was carried out over the long period of time for the purpose of establishment of a fowl precursor adipose cell stock, and the comparative examination of the growth and differentiation potency between passages was carried out.

[0052]1) The subculture method of material and the method CPMA was performed according to (2) of Example 3. That is, after removing CPMA from the culture dish bottom and carrying out centrifugal washing using trypsin EDTA-PBS, the cell number was computed using the erythrocytometer. It was made to re-float by the FCS addition DMEM 10% so that it may be set to a last concentration 1x10 ^{5} individual / ml, 2 ml of cell suspension was moved to the culture dish, and it cultivated for eight days within the CO'2 incubation device under the gaseous phase of 37 **, 5%CO_2, and 95% air. Culture-medium exchange was performed day by day [4], and the above operation was repeated for every passage. About observation of the proliferation state, it observed every day using the inverted microscope. About differentiation inducing, it carried out by exchanging the culture medium of CPMA attained confluent eight days after subculture for the differentiation-inducing culture medium shown in (2) of Example 4. Then, it cultivated for 12 days in the differentiation-inducing culture medium further. CPMA was sampled 12 days after differentiation inducing, and the G3PDH specific activity value was measured in accordance with the method shown in (2) of Example 2. Observation of the cell form change after differentiation inducing was performed every day using the inverted microscope.

[0053]2) Join Although CPMA in ******** showed the gestalt like a smooth muscle cell a little compared with the swine precursor adipose cell of Example 3, it was not observed about the morphological difference between each passage. The influence of the number of times of a passage exerted on growth of CPMA was shown in drawing 10. Only in CPMA of two examples,

reduction was accepted in the cell number eight days after culture of the 10–15th generation of the passage among five examples. However, in the other CPMA, the same proliferation potential power as the early stages of a passage was maintained also in the 33rd generation of the passage.

[0054]As for the shape change of CPMA observed after differentiation inducing, the difference was not observed between each passage. Among five examples, by CPMA of two examples, even if it repeated the passage of 33 or more generation, after differentiation inducing, the lipid droplet of various sizes was accumulated into cytoplasm, and the morphological difference with the early stages of a passage was not accepted. The influence of the number of times of a passage exerted on differentiation of CPMA was shown in drawing 11. By CPMA of two examples, the G3PDH specific activity value was maintained to the 33rd generation of the passage among five examples. However, the G3PDH specific activity value in other CPMA(s) of three examples, It decreased quickly from the passages 6 and 8 and the 25th generation, respectively, and each of passages 8 and 9 and G3PDH specific activity values after the 29th generation fell to the same value (6 – 18units/mg protein) as the control plot which does not carry out differentiation inducing, respectively. Even if CPMA of unilocular fat cell origin passes through long-term passage, the above result shows that uniform growth and the differentiation characteristic are maintained, and requires it. Therefore, CPMA became clear [that it is a fowl precursor adipose cell stock in which a long-term passage culture is possible].

[0055]

[Effect of the Invention]It is as follows when the effect acquired by this invention is listed.

[0056]1) It was shown clearly that the unilocular fat cell which accomplished end differentiation was dedifferentiated even to a precursor adipose cell. This can serve as an experiment system for a mechanism break through with the gene level of not only the model of adipocyte differentiation but differentiation and dedifferentiation.

[0057]2) Long-term passage is possible without transformations (abnormalities in a chromosomal level, etc.) for the precursor adipose cell of these. That is, it becomes possible to establish the precursor adipose cell stock of animal cell origin with reproducibility sufficient for a short period of time simple by this invention.

[0058]3) If the substance which controls growth and differentiation can be screened using livestock or the precursor adipose cell stock of domestic-fowls origin, it will become controllable [more effective body fat accumulation] by medicating livestock or domestic fowls with it. It decreases the further improvement in a feed efficiency, and the fat discarded, and it not only solves the limit of the conventional method by the regulation of feed energy or a nutrient performed by this method now, but contributes to the increase in efficiency of food resources.

[0059]4) It is shown clearly that fat tissue has the characteristic which is different to every [which is formed] parts (a mesentery, the kidney circumference, hypodermic, an epididymis, the muscular system, etc.). Since the precursor adipose cell of each part origin is extracted for every each object by this invention, it becomes possible to carry out the comparative examination of the characteristic of the adipocyte differentiation for every part in detail including individual difference.

[0060]5) Although the fat (marbled meat) crossed in the muscular system raises the commodity value of meat, if it establishes the precursor adipose cell stock of muscular system origin and those characteristics are solved by this invention, it will enable high meat production of commodity value.

[0061]6) Use the precursor adipose cell of internal-organs origin, and it becomes an experiment system for a break through of mechanisms of production, such as TNFalpha [which participates in the physiological active substance generated and secreted especially obesity, or an adult disease deeply], leptin, and PAI-1, from this cell. Thereby, development of insulin resistance and obesity dissolution medicine, making the index of arteriosclerosis, etc. become possible.

[0062]7) Since a single precursor adipose cell is obtained by this invention, it becomes possible to investigate about a mature fat cell, blood vessel epithelial cells or the pericyte, and the influence that cocultivation with a muscle cell etc. of is still attained, and those cells have on adipocyte differentiation.

[0063]8) Endocrine disrupting chemicals (environmental hormone), such as dioxin or a synthetic resin, are lipophilicity, and are accumulated in fat tissue. Therefore, it becomes incorporation of those substances and an experiment system which examines the mechanism of discharge in detail using the fat cells which carried out differentiation inducing.
[0064]9) setting fat cells in a differentiation process — fat cells — since a specific gene is revealed tidily, it can be caught clearly which position of a differentiation process it is a cell in. Therefore, the arbitrary cells which result in end differentiation further metaphase and the second half can be prepared in a proliferation period and the first half of differentiation. If it is with this invention, it is possible from a fetal period to prepare the precursor adipose cell of adult origin easily, and since it is maintained without causing a chromosomal aberration, it can be used as a donor cell at the time of somatic cell clone creation.
[0065]10) this invention can obtain a precursor adipose cell, if a mature fat cell is extractable — mainly — spittle — it was shown for milk and birds. This can build the experiment system and matter production system for investigating the differentiation characteristic of the fat cells in reptiles, an amphibian or fishes, and an invertebrate, for example, Insecta etc., etc.
[0066]

[Translation done.]

* NOTICES *

1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

CLAIMS

[Claim(s)]
[Claim 1]A precursor adipose cell stock of this animal origin acquired by carrying out subculture of the fibroblast Mr. fat cells formed by carrying out ceiling culture of the unilocular fat cell of an animal, and carrying out differentiation inducing.
[Claim 2]The precursor adipose cell stock according to claim 1 whose animal is Homo sapiens.
[Claim 3]The precursor adipose cell stock according to claim 1 whose animal is a swine.
[Claim 4]The precursor adipose cell stock according to claim 1 whose animal is a fowl.

[Translation done.]

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(54) PRECURSOR ADIPOCYTE STRAIN

(57)Abstract:
PROBLEM TO BE SOLVED: To simply obtain the subject animal-derived adipocyte strain useful for the production of a meat having a high commercial value, the development of insulin-resistant antidiabetic medicines, antiobestic medicines, etc., in short periods in good reproducibility by subculturing a specific fibroblast-like adipocyte to induce the differentiation of the cell.
SOLUTION: This animal-derived precursor adipocyte is obtained by subculturing a fibroblast-like adipocyte to induce its differentiation without causing transformation. The fibroblast-like adipocyte is obtained by adhering the unicellular fat cell of an animal such as human, swine or fowl, for example, to the inside upper surface (ceiling surface) of a flask fully filled with a culture medium and subjecting the cell to a ceiling culture.